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Official Report of Patent Disclosure

[Name of the invention]

Peptide compound containing high level of glutamine, the manufacturing process and an enteral nutrient

[Abstract]

Composition: A peptide compound containing glutamine and/or glutamic acid (more than 40%) which is obtained by protease treatment of gluten or zein. Molecular weight of this peptide distributes between 131-1141 dalton (by gel filtration method).

Effect: The peptide has a suppressive function of atrophy of intestinal mucosa, and is available for an enteral nutrient or an ingredient of transfusion.

[Scope of patent claim]

[Claim term 1] The amino acid composition of this peptide compound is more than 40% glutamine and glutamic acid.

[Claim term 2] The peptide compound described in *Claim term 1*, which the free amino acid content is less than 10% and the average amino acid chain length is 2 to 5.

[Claim term 3] A peptide complex, composed of di-, tri-, tetra- and pentapeptides, and containing small proportion of free amino acids. It has following properties.

- 1) The average molecular weight is approximately 200 dalton (estimated by gel filtration method).
- 2) Distribution of molecular weight: The molecular weights are mainly distributed between 131 and 1411 dalton, and the distribution peak is around 200 dalton (estimated by gel filtration method).
- 3) More than 40% of the hydrolysate amino acid is quantified as glutamic acid.
- 4) Total amount of free amino acids of this compound is less than 10%, and free glutamic acid is less than 5%.
- 5) It has a suppressive function of atrophy of intestinal mucosa.

[Claim term 4] A manufacturing method of the peptide compound composed of more than 40% of glutamine or glutamic acid. This method is characterized by following procedure: a material protein composed of more than 20% glutamine is digested by one or more kinds of peptidases, and free amino acids are removed, and then, the peptide is fractionated.

[Claim term 5] A manufacturing method described in *Claim term 4*, which the material protein is gluten or zein.

[Claim term 6] An enteral nutrient which mixed with the peptide compound described in Claim term 1 as a glutamine source, and which characterized by the suppressive effect of an atrophy of intestinal mucosa.

[Explanation of the invention]

<<<<<<<<< [0001] - [0013] were eliminated. >>>>>>>>>

[0014] Following description is examples of operation and experiment to explain the detail of the invention.

[An example of the operation]

Production of peptide compound containing high level of glutamine

1) Preparation of material protein

Lipid was removed from 10 g of wheat gluten by ethylether. After discard of ethylether, the sample was suspended in 200 ml of 1% acetic acid solution. The solution was homogenized vigorously using mixer, the homogenate was centrifuged at 500 x g for 10 min. Protein concentration of the supernatant was measured by Protein-Assay Kit (Biolad). Then, it was diluted by 1% of acetic acid to make 1% of protein solution.

[0015] 2) Chose of enzyme

Gluten suspended in aqueous phase reacts easily with enzyme under acidic or alkaline condition. Since Pyrrole reaction (N-terminal cyclic reaction) occurs under the alkaline condition, we used acidic protease as an enzyme which hydrolyze a protein. Molsin (Sigma, Co.), pepsin (Sigma, Co.) or rapidase (Takeda Chemical Co.) were reacted individually with gluten for 24 hrs at 37°C. A distribution of product's molecular weight was measured by Biogel P-2 gel (Biolad) filtration. Sample (50 mg) was dissolved in 0.1 M phosphate buffer (pH 7.0), and put on the Biogel P-2 column (1.6 x 87.5 cm). The sample was eluted by the 0.1 M phosphate buffer at flow rate of 19 ml/hr. A void volume of the column was 48 ml. Bacitracin (MW 1411), Isoleucylglycine (MW 188), and Isoleucine (MW 131) were used as molecular weight markers. As a result, a product reacted with molsin gave the lowest molecular weight.

[0016] 3) Enzymatic treatment

The molsin (enzyme) was reacted with gluten (substrate) in 1% acetic acid solution at the enzyme/substrate ratio of 1:100 for 24 hrs at 37°C. Since almost all molecular weight of the reaction product is higher than 1,000 dalton, the sample was hydrolyzed more by molsin. Then, the pH was adjusted to 7.0, and actinase E (Kaken Pharmaceutical Co.) was reacted with the product. The reaction with actinase E was exactly same condition as that with molsin except pH. The hydrolyzed product was measured by Biogel P-2 gel filtration and the result was shown in Fig. 1. The values (1411, 188 and 131) expressed the elution position of molecular weight markers. The result showed that the absorbance had a molecular weight peak around 188.

[0017] 4) Condition of actinase treatment

The amount of free amino acid after the different periods of actinase treatment was measured and shown in Table 1. The high level of free amino acids were in order that Gln > Val > Phe > Ile. However, the percentages of the amino acids which originally come from gluten were Glx 9.6%, Val 83.4%, Phe 67.4% and Ile 74.4%. It was assumed that a large part of Gln remained as peptide form, whereas more than 67% of Val, Phe and Ile were set free. Since all amino acids except Glu were selectively set free by actinase treatment, Gln content of the peptide product was much higher than that of the material gluten.

[0018] Table 1

Table 1. Time Course Change of the Amount of Free Amino Acids^a by Actinase Treatment

Amino acids	Reaction time (hr)						b hydrolysate (H)	c Gluten (G)	d Free form (%)
	0	1	3	7	24	40			
Asp	0.0	0.6	2.2	4.4	11.6	11.6	8.9	199.3	68.1
Asn	0.0	23.8	39.0	72.5	107.8	124.3	7.4	-	-
Thr	0.0	5.0	13.7	25.0	51.8	81.2	5.0	213.9	26.6
Ser	0.0	12.7	40.6	84.5	165.6	165.6	8.6	497.2	35.0
Glu	0.0	2.9	4.8	8.3	19.0	30.6	17.3	2869.8	9.6
Gln	0.0	36.1	93.7	219.7	222.6	250.9	17.5	-	-
Gly	0.0	2.7	7.9	14.6	34.8	59.9	3.6	411.5	9.3
Ala	0.0	20.5	54.5	95.2	151.1	151.9	15.4	244.8	68.0
Val	0.0	63.4	111.7	152.4	208.2	211.1	9.1	260.4	83.4
1/2 Cys	0.0	2.4	3.0	3.7	13.4	23.9	2.0	108.5	14.2
Met	0.0	59.2	82.1	91.2	96.8	95.6	1.1	109.8	89.2
Ile	0.0	66.4	131.9	159.4	163.0	174.6	10.1	232.6	74.4
Leu	0.0	35.2	38.6	38.6	41.5	42.6	110.9	503.9	30.2
Tyr	0.0	88.2	107.3	129.1	138.4	140.4	15.6	188.1	81.9
Phe	0.0	126.6	153.7	175.8	195.8	202.7	40.8	351.1	67.4
Lys	0.0	5.4	10.8	16.8	32.4	50.8	16.8	134.4	36.7
His	0.0	13.7	29.0	51.6	86.9	101.0	3.1	138.1	65.2
Arg	0.0	4.7	10.4	15.8	26.5	26.5	32.3	84.3	69.8
Pro	0.0	19.8	32.5	66.7	95.3	126.0	1.0	1198.0	8.0
Total	0.0	589.3	967.3	1425.3	1862.0	2162.5	326.5	7745.7	28.3

a: nmol obtained from 1 mg of hydrolysate
b: Free amino acid by molsin treatment

c: Amino acid content in 1 mg of gluten
d: (value of 24 hr + H) x 100 / G

[0019] 5) Remove of free amino acids

Following Yamashita's method (Yamashita, et al. J. Food Sci., 41, 102, 9, 1976), the sample was filtered using Sephadex G-15 column (2.5 x 60 cm, Phalmacia) to remove

free amino acids from the peptide. It was eluted by 10% ethanol (pH 7.0) at the flow rate of 95 ml/hr. The void volume of the column was 96 ml. The chromatogram was shown in Fig. 2. It was possible to be divided into 4 fractions. We called them as Fr. 1, 2, 3 and 4, respectively. The amino acid profile of each fractions was shown in Table 2.

[0020] Table 2

Table 2. Amino Acid Composition of Hydrolysate (% weight)

Amino acids	Fr.1	Fr.2	Fr.3	Fr.4	Gluten
Asx	2.99	2.50	ND	ND	2.59
Thr	1.79	3.66	0.0	0.0	2.46
Ser	3.28	7.99	12.4	2.8	4.93
Glx	50.33	39.11	63.2	17.5	41.83
Gly	3.63	3.97	ND	2.3	2.67
Ala	0.88	4.82	0.0	ND	1.98
Val	1.77	7.03	0.0	0.0	2.94
1/2 Cys	2.12	0.00	0.0	0.0	1.27
Met	0.37	0.00	0.0	0.0	1.64
Ile	1.63	4.33	0.0	3.0	2.99
Leu	2.25	6.34	24.4	6.4	6.49
Tyr	0.93	0.00	ND	0.0	3.49
Phe	2.50	ND	ND	48.7	5.88
Lys	1.49	3.75	0.0	0.0	1.96
His	1.04	ND	0.0	2.6	2.15
Arg	1.97	7.32	0.0	0.0	1.5
Pro	20.82	9.17	ND	14.8	12.99
Trp	0.20	-	-	-	0.24

[0021] Glx content was high in Fr. 1 and Fr. 3. The result of free amino acid analysis showed that almost all Glx in Fr. 3 was free form. On the other hand, only 3.5% of Glx in Fr. 1 was free form, indicating that almost all Glx in Fr. 1 was peptide form. Also, Glx content of Fr. 1 was higher than that of the material gluten.

[0022] 6) Analysis of N-terminal glutamine

We analyzed N-terminal amino acid of Fr. 1. Following Hartley's method (Biochem. Biophys. Acta, 21, 58, 1956), peptide was dansylized and then hydrolyzed at 110°C for 24 hrs. The amino acid profile was analyzed. The amount of Glx which was not dansylized was defined as "D". The amount of Glx when the same amount of sample was dansylized was defined as "Q". The amount of Glx which existed at N-terminal was calculated from "Q - D". As a result, 17.6% of total Glx existed at N-terminal of Fr. 1. The proportion of Glx existed in any other portion except N-terminal, which was

stable in aqueous phase was 78.9% (=100-3.5-17.6). In Fr. 1, Glx composed 50.33% of total amino acids, but the proportion of Glx which was stable in aqueous phase was estimated to 39.71%. Peptide produced from gluten was mainly consisted from glutamine.

[0023] 7) Distribution of molecular weight of peptide compounds

Fr. 1 (50 mg) was dissolved in 0.1 M phosphate buffer (pH 7.0) and put on a Biogel P-2 column (1.6 x 87.5 cm). The sample was eluted by the same buffer at flow rate of 19 ml/hr. The void volume of the column was 48 ml. As molecular weight markers, we used bicitracin (MW 1411), isoleucylglycine (MW 188) and isoleucine (MW 131). The chromatogram was shown in Fig. 3. The major peptide compounds were tetra- and penta peptide. Also, 88% of them were peptide or amino acid. The recovery was 35% of the material protein, based on the nitrogen amount.

[0024] [An example of the experiment]

Effects of glutamine peptide on intestinal functions

We showed here an example which investigates the effect of peptide compounds obtained from "An example of the operation" on intestinal functions.

1) Materials and methods

Diet composition: β -cornstarch, cellulose powder, soybean oil, choline chloride, casein, mineral mix. (Harper) and vitamin mix. (Harper) were used to prepared the purified diet. Rats were divided to 5 groups according to the difference of nitrogen source, and fed the diets for 7 days. The diet composition of each groups was shown in Table 3.

[0025] [Table 3]

Table 3. Diet Composition^a

Ingredients	Groups				
	Control	Free glutamine	Gluten	Glutamine peptide	Amino acid mix
Starch	30.4	30.4	30.4	30.4	30.4
Soybean oil	2.1	2.1	2.1	2.1	2.1
Cellulose	2.1	2.1	2.1	2.1	2.1
Mineral mix.	1.7	1.7	1.7	1.7	1.7
Vitamin mix..	0.4	0.4	0.4	0.4	0.4
Choline	0.0625	0.0625	0.0625	0.0625	0.0625
Casein	5.00	5.00	5.00	5.00	5.00
Glutamine	-	1.25	-	-	-
Gluten	-	-	2.83 ^b	-	-
Peptide	-	-	-	2.83 ^b	-
Amino acid mix	-	-	-	-	2.48 ^b
Lys-HCl	-	-	0.02	0.02	0.02

a: g/kg/day b: estimated to 1.25 g of Gln

[0026] Experimental animals: Male Wistar rats (150g) were purchased from Charles River. After a certain period of acclimatization, they were used for the feeding experiment. They were fasted for first 4 days to make a malnutrition animal model, and they were fed the purified diets for next 7 days (meal feeding). The animals were killed 18 - 24 hrs after the final feeding. We used methotrexate (MTX, Wako Pure Chemical Co.) to make a intestinal function damaged model rat. Four days after the feeding, we started to inject MTX (20 mg/kg) by peritoneoclysis, following Fox's method (Surgical Forum, 38, 43, 1987). Then, the rats were fed the diet for next 3 days (total 7 days), and killed 18 - 24 hrs after the final feeding. All rats were killed between 12:00 - 15:00 to avoid the effect of the diurnal variations. The rats drunk water freely. Three or 4 rats were used for each experiments. The initial body weight of rats were comparable between the experimental groups.

[0027] Intestine: The rats were anesthetized with sodium pentobarbital (Dai Nippon Pharmaceutical Co.) and intestine was excised. After contents of intestine was washed out with ice-cold saline, total weight of intestine was weighed. Second 10 cm from the top edge of it was cut, and the intestinal mucosa was exfoliated from this part using slide glass. It was homogenized with 19-fold volume of ice-cold saline for 30 sec, and the homogenate was centrifuged at 3,000 x g for 10 min. The supernatant was used for following assays.

[0028] Quantitation of mucosal protein: It was measured using Protein Assay Kit (Biolad).

Mucosal sucrase activity: Substrate sucrose solution (0.056 M) was prepared with 0.1 M maleate buffer (pH 6.0). Sample solution (0.1 ml) and one drop of toluene were added to 0.1 ml of the substrate solution, and reacted for 30 min at 37°C. After the reaction, 1 ml of distilled water was added to it, and it was boiled for 2 min. After cooling down to room temperature, produced glucose was quantified by a commercial kit (Glucose C-II - Test Wako, Wako Pure Chemical Co.). Enzymatic activity was expressed as a unit of μ mol sucrose hydrolyzed / 60 min / mg protein.

[0029] Mucosal amino peptidase activity: Sample solution was reacted with 0.5 mM Leu-NA for 5 min under the 100mM potassium phosphate buffer (pH 8.0) containing 1mM dithiothreitol. The reaction was terminated by adding 0.4 ml of 0.23 N HCl-EtOH. Then, 0.4 ml of ethanol containing 0.06% *p*-dimethyl cinnamic aldehyde was added and left for 30 min. The amount of the produced schiff's base pigment was measured at 540 nm to quantify the free β -naphthylamine. The enzyme activity was expressed as a unit of ΔA_{540nm} / min / mg protein. Statistical analysis: t test.

[0030] 2) Results

Effect of malnutrition on intestinal function in rats was shown in Table 4.

Table 4. Body Weight Change, Weight of Intestine, Mucosal Protein Level and Intestinal Enzyme Activity in Malnutrition Rats

	Control	Free Gln	Gluten	Amino acid mix	Glutamine peptide
Body weight change ^a	-6.9 ± 6.1	-3.4 ± 1.4	4.8 ± 5.2	-2.0 ± 11.1	0.9 ± 9.7
Weight of intestine (g)	2.9 ± 0.3	3.4 ± 0.2	3.4 ± 0.1*	3.4 ± 0.6	3.7 ± 0.3*
Mucosal protein ^b	156 ± 11	166 ± 15	165 ± 15	169 ± 10	179 ± 5*
Sucrase activity ^c	110 ± 8	101 ± 11	87 ± 17	86 ± 18	96 ± 22
Aminopeptidase activity ^d	4.6 ± 0.3	6.0 ± 0.9	5.9 ± 0.9	6.1 ± 0.6	6.0 ± 0.4

a: g / 7 days b: mg / g wet tissue

d: ΔA540nm / min / mg protein

c: μmol sucrose hydrolyzed / 60 min / mg protein

*Significantly different at p < 0.05.

[0031] In malnutrition rats, body and intestine weights were significantly increased only by the supplementation of gluten or glutamine peptide. The protein level of jejunum mucosa in the glutamine peptide group was significantly higher than that in the control group, whereas that in the amino acid mixture group which represented the identical amino acid composition was comparable to that in the control group. It suggests that the invented peptide is superior to amino acid mixture in the activation of intestinal functions.

[0032] Effects of MTX on intestinal function in rats were shown in Table 5.

Table 5. Body Weight Change, Weight of Intestine, Mucosal Protein Level and Intestinal Enzyme Activity in MTX Treated Rats

	MTX treated groups					
	Normal	Control	Free Gln	Gluten	Amino acid mix	Glutamine peptide
Body weight change ^a	-	-5.0 ± 3.5	-0.3 ± 6.5	1.0 ± 7.4	1.6 ± 9.9	0.9 ± 6.3
Weight of intestine (g)	4.9 ± 0.8	2.8 ± 0.6	2.6 ± 0.3	3.3 ± 0.7	2.6 ± 0.4	3.1 ± 0.5
Mucosal protein ^b	146 ± 10	103 ± 21	126 ± 1	119 ± 17	100 ± 8*	118 ± 4
Sucrase activity ^c	57.7 ± 2.1	19.4 ± 0.6	33.3 ± 6.8*	26.9 ± 5.6	22.6 ± 3.5	36.5 ± 3.9**
Aminopeptidase activity ^d	3.2 ± 0.1	2.1 ± 0.1	2.3 ± 0.3	2.4 ± 0.2	1.9 ± 0.2	2.5 ± 0.0*

a: g / 7 days b: mg / g wet tissue c: μmol sucrose hydrolyzed / 60 min / mg protein

d: ΔA540nm / min / mg protein *, **Significantly different at p < 0.05 and p < 0.01, respectively.

[0033] It is known that a lesion of intestinal villas reaches peak 3 days after the MTX administration. Also, 20 mg/kg of the MTX administration kills the animals (100%) within 156 hrs. Fox, et al. have reported that the administration of glutamine (Free form) suppresses significantly the lesion caused by MTX (Surgical Forum, 38, 43,1987). Compared with the normal rats, the weight of intestine and the protein content of jejunum was decreased to 57% ($p < 0.05$) and 71% ($p < 0.05$), respectively, by MTX administration. Also, the MTX administration suppressed significantly the sucrase and leucine amino peptidase activities ($p < 0.01$). Approximately 25% of the MTX administrated rats had diarrhea. On dissection, a lot of digested food remained in the stomach and intestines in the MTX administrated rats but in the malnutrition rats. Therefore, it was interpreted that the intestinal function was damaged by the MTX administration.

[0034] The supplementation of glutamine peptide to the diet increased the sucrase activity ($p < 0.01$) and the leucine amino peptidase activity($p < 0.05$) in the MTX rats. The same effect was observed in the free glutamine supplementation group. However, the effect of amino acid mixture was not observed. It indicated that a resistibility against a lesion of intestinal function was stronger in the peptide absorption system than in the amino acid absorption system. It was confirmed that the effect of glutamine on intestine was more potent in the peptide form than in the free form.

[0035]

[Effect of the invention] The execution of this invention gives a peptide compound containing high level of glutamine and an enteral nutrient with this peptide compound. This peptide compound is stable in aqueous phase, because N-terminal glutamine does not bond. It suppresses the atrophy of intestinal mucosa which observed in malnutrition. Also, the peptide compound blended as an external nutrient increases the glutamine absorption.

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Ext. No. 44052

[Figure legends]

[Fig. 1] Chromatogram of Biogel P-2 gel filtration chromatography of actinase treatment sample obtained from the acidic protease treatment of gluten. It was measured the absorbance at 260nm as an index of peptide concentration.

[Fig. 2] Chromatogram of the solution which was removed free amino acids from the sample (described in Fig. 1) by Sephadex G-15. It was measured the absorbance at 260nm as an index of peptide concentration.

[Fig. 3] Biogel P-2 gel filtration chromatogram of the peptide compound (Fr. 1 in Fig. 2). It was measured the absorbance at 260nm as an index of peptide concentration.

Fig. 1

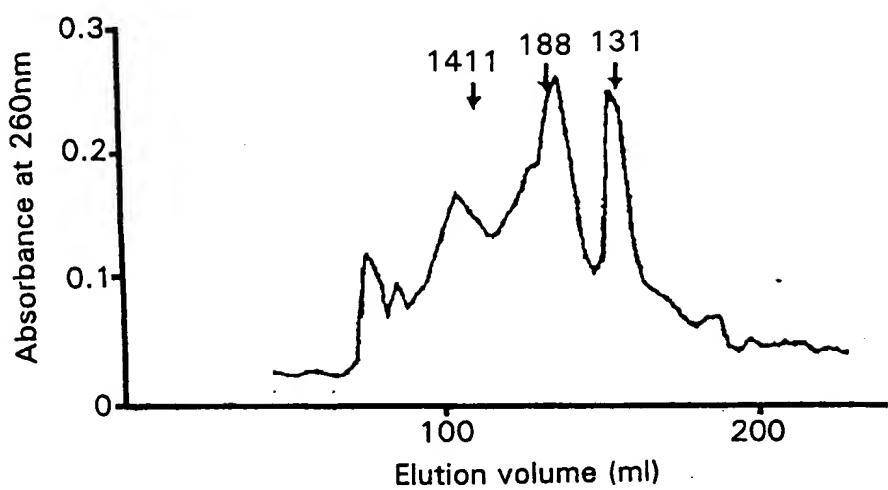


Fig. 2

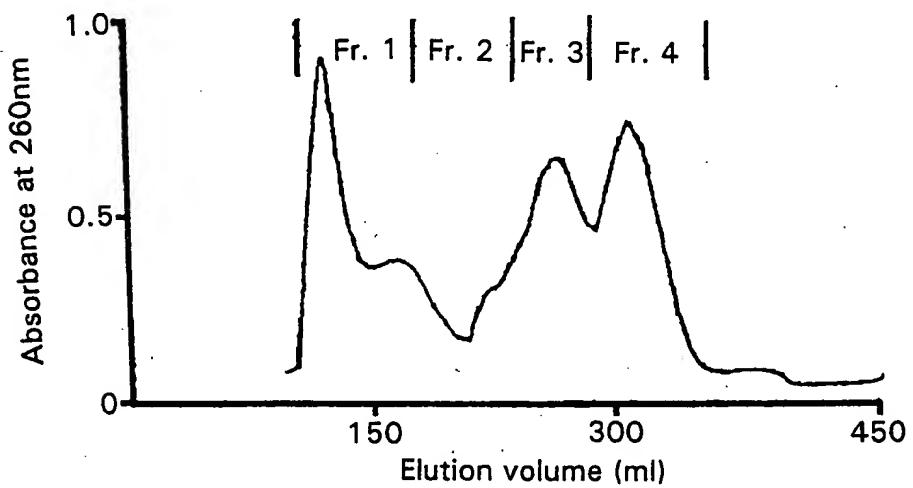
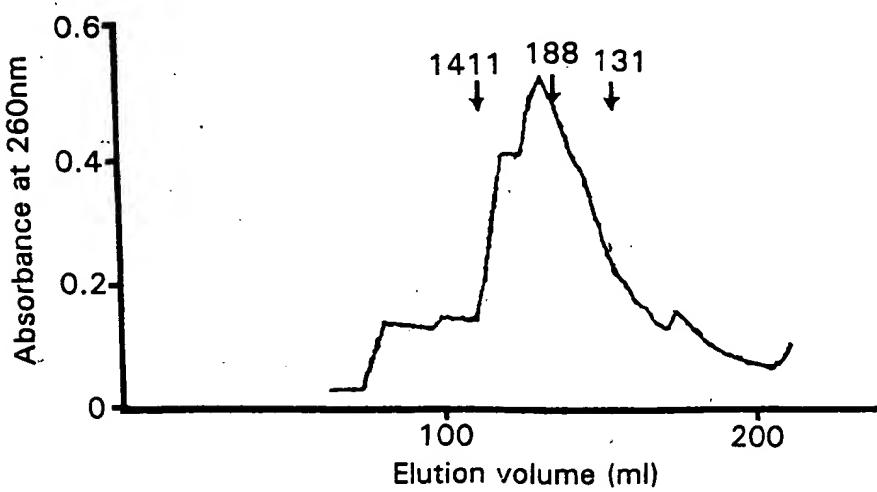


Fig. 3



Tokukai Kohi 5-236909

Japanese Patent Application Laying-open (KOKAI) No. 5-236909
laid open September 17, 1993

Application No. 4-78250 filed February 28, 1992 ←

Applicant: Show Brand Milk K.K., Sapporo, Japan

Inventors: S. Araki, et al, Japanese citizens

↑
prior art
date

Title of Invention: Glutamine-rich peptide compositions,
process for the preparation thereof, and
enteral nutrition

Abstract:

A peptide composition containing 40% or more, based on the constituting amino acids, of glutamine or glutamic acid obtained by treating a gluten or sein with a protease, the molecular weight thereof being distributed in the range of 131 to 1141 daltons as measured by gel filtration method.

The composition has a function of inhibiting the degradation of enteric mucosa and may be utilized as an ingredient in enteral nutrition or infusion.

Claims:

1. A peptide composition containing 40% or more, based on the constituting amino acids, of glutamine or glutamic acid.
2. The peptide composition of claim 1, wherein the content of free amino acids in the peptide composition is 10% or less, and the average chain length of the peptide comprises 2 to 3 amino acids.
3. A peptide composition which is obtainable by hydrolysing a gluten or sein with an acidic protease and a neutral protease successively and subjecting the hydrolysate to chromatography, which is composed of di- to pentapeptides and further contains a small amount of free amino acids, and which has the following properties:
 - (1) Average molecular weight being about 200 daltons (gel filtration);
 - (2) Molecular weight distribution being such that the major proportion thereof is distributed in the range of 131 to 1411 daltons and there is a peak near 200 daltons (gel filtration);
 - (3) 40% or more of the amino acids after the hydrolysis being quantitatively determined to be glutamic acid;

(4) Free amino acid content being 10% or less and free glutamic acid content being 5% or less; and
(5) Having a function of inhibiting the degradation of enteric mucosa.

4. A process for preparing a peptide composition containing 40% or more of glutamine or glutamic acid as the constituting amino acid, comprising decomposing a protein containing 20% or more of glutamine as the constituting amino acid with one or more proteolytic enzymes, removing free amino acids, and preparatively separating a peptide fraction.

5. The process of claim 4, wherein the protein is a gluten or zein.

6. An enteric nutrition containing a peptide composition of claim 1 as a glutamine source and having a function of inhibiting the degradation of enteric mucosa.

PEPTIDE COMPOSITION HAVING HIGH GLUTAMINE CONTENT, ITS PRODUCTION AND ENTERAL FEEDING AGENT

Patent Number: JP5236909
Publication date: 1993-09-17
Inventor(s): ARAI SOICHI; others: 02
Applicant(s): SNOW BRAND MILK PROD CO LTD
Requested Patent: JP5236909
Application Number: JP19920078260 19920228
Priority Number(s):
IPC Classification: A23L1/305 ; A61K37/02 ; C12P21/06
EC Classification:
Equivalents: JP2524551B2

Abstract

PURPOSE:To obtain the subject composition effective in suppressing the degeneration of the mucous membrane of the small intestine, having excellent stability and glutamine-absorbency and useful as a raw material for infusion, etc., by decomposing a specific protein with a proteinase, removing free amino acids and collecting a peptide fraction.

CONSTITUTION:A protein containing >=20% of glutamine as a constituent amino acid is decomposed with one or more kinds of proteinases. Free amino acids are removed from the decomposition product and a peptide fraction having molecular weight distributing within the range of 131-1,141 dalton (determined by gel permeation) is collected to obtain the objective composition containing glutamine or glutamic acid in an amount accounting for >=40% of the constituent amino acids. An enteral feeding agent can be prepared by compounding the composition as a glutamine-source.

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(19)日本国特許庁 (J P)

(12) 公開特許公報 (A)

(11)特許出願公開番号

特開平5-236909

(43)公開日 平成5年(1993)9月17日

(51)Int.Cl.⁵
A 2 3 L 1/305
A 6 1 K 37/02
C 1 2 P 21/06

識別記号 庁内整理番号
8314-4C
8214-4B

F I

技術表示箇所

審査請求 未請求 請求項の数 6 (全 11 頁)

(21)出願番号

特願平4-78260

(22)出願日

平成4年(1992)2月28日

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(54)【発明の名称】 グルタミン含量の高いペプチド組成物、その製造方法及び経腸栄養剤

(57)【要約】

【構成】 グルテンあるいはゼインをプロテアーゼで処理して得られるグルタミンもしくはグルタミン酸を構成アミノ酸の40%以上含有し、分子量が131~1141ダルトン(ゲル濃過法による)の範囲に分布するペプチド組成物。

【効果】 小腸粘膜退化抑制機能を有し、経腸栄養剤あるいは輸液の成分として利用することができる。